

Short communication

Genotyping studies of *Toxoplasma gondii* isolates from Africa revealed that the archetypal clonal lineages predominate as in North America and Europe

G.V. Velmurugan^a, J.P. Dubey^{a,*}, C. Su^b^a United States Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Animal Parasitic Diseases Laboratory, Building 1001, Beltsville, MD 20705-2350, USA^b Department of Microbiology, The University of Tennessee, Knoxville, TN 37996-0845, USA

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Abstract

Until recently, *Toxoplasma gondii* was considered to be clonal with very little genetic variability. Recent studies indicate that *T. gondii* isolates from Brazil are genetically and biologically different from *T. gondii* isolates from USA and Europe. However, little is known of the genetics of *T. gondii* strains from Africa. In this study, we genotyped 19 *T. gondii* isolates from chickens from six African countries (Egypt, Kenya, Nigeria, Congo, Mali, and Burkina Faso) using 10 PCR-RFLP markers (SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico). The results revealed four genotypes. Thirteen isolates belong to the Type III lineage, five isolates have Type II alleles at all loci except apico and they belong to the Type II lineage. One isolate from Nigeria had atypical genotype. In general, these isolates were mostly clonal Type III and II strains that predominate in North American and European. DNA sequencing at several loci for representative isolates confirmed the results of PCR-RFLP genotyping. Taken together with recent studies of *T. gondii* isolates from Africa, it is clear that the three clonal lineages (Types I, II and III) predominate not only in North America and Europe, but also in Africa.

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Keywords: *Toxoplasma gondii*; Chickens; Genotype; PCR; Africa

1. Introduction

Toxoplasma gondii infections are widely prevalent in human beings and other animals worldwide (Dubey and Beattie, 1988). Humans become infected post-natally by ingesting tissue cysts from undercooked meat, consuming food or drink contaminated with oocysts, or by accidentally ingesting oocysts from the environment. However, only a small percentage of exposed adult

humans or other animals develop clinical signs of disease. Whether the severity of toxoplasmosis in immunocompetent hosts is due to the parasite strain, host variability, or to other factors is largely unknown. Recently, attention has been focused on the genetic variability among *T. gondii* isolates from apparently healthy and sick hosts. In humans in French Guiana and Suriname, severe cases of toxoplasmosis in immunocompetent patients have been related to *T. gondii* strains with unusual genetic characteristics (Ajzenberg et al., 2004; Demar et al., 2007).

Most *T. gondii* isolates from human and animal sources have been grouped into one of three clonal lineages (Types I, II, and III) by multi-locus enzyme

* Corresponding author. Tel.: +1 301 504 8128; fax: +1 301 504 9222.

E-mail address: jitender.dubey@ars.usda.gov (J.P. Dubey).

electrophoresis, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and microsatellite typing (Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg et al., 2002a,b). We have recently found that the isolates of *T. gondii* from Brazil are biologically and genetically different from those in North America and Europe (Dubey et al., 2002; Lehmann et al., 2006; Dubey et al., 2007a,b,c). *T. gondii* isolates from asymptomatic chickens from Brazil were more pathogenic to mice than isolates from Europe or North America, irrespective of the genotype (Dubey et al., 2006). Additionally, most isolates from chickens from Brazil were not clonal, and Type II was absent (Dubey et al., 2007a).

Little is known of the genetic characteristics of *T. gondii* isolates from Africa, and the information is mostly based on a few PCR-RFLP markers. We have initiated a study to describe the biological and genetic characteristics of *T. gondii* isolates from chickens from Africa (Dubey et al., 2003, 2005; Lehmann et al., 2006; Lindström et al., 2008). In the present paper, we genotyped 19 *T. gondii* strains using 10 PCR-RFLP markers to achieve a high resolution in identification.

2. Materials and methods

For the present study, 19 *T. gondii* isolates (Table 1) from six countries were investigated. Isolation procedures for the 18 strains, except the isolate from Nigeria, were described previously (Dubey et al., 2003, 2005). Briefly, chicken tissues were imported from these countries, bioassayed in mice, and tachyzoites or bradyzoites obtained from these isolates were cryopreserved in liquid nitrogen on their primary isolation. For the present study, cryopreserved material was inoculated subcutaneously into two out-bred female Swiss Webster mice (Taconic Farms, Germantown, NY) as described by Dubey et al. (2002). The recipient mice were tested for *T. gondii* infection. Tissue imprints of brains or lungs of mice that died were examined for *T. gondii* tachyzoites or tissue cysts. Survivors were bled on day 41 post-inoculation (p.i.) and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies with the modified agglutination test (MAT). Mice were killed six weeks p.i. and brains of all mice were examined for tissue cysts as described (Dubey and Beattie, 1988).

The Nigerian isolate was obtained in 2005. Seventy-nine chickens were purchased from a market in Vom, Plateau, Nigeria. The chickens were killed on January 24, 2005, and their serum and hearts were sent cold by air to Beltsville, MD. Serum samples were tested for

antibodies to *T. gondii* by the MAT with serum dilutions of 1:10–1:40 (Dubey and Desmonts, 1987). Five chickens were seropositive with MAT titers of 1:10 in three, 1:20 in one, and 1:40 in one chicken. Hearts of each of the five seropositive chickens were homogenized, digested in pepsin, and the digest inoculated subcutaneously into five mice as described (Dubey et al., 2002). *T. gondii* was isolated from the heart of one chicken (no. 22) with a MAT titer of 1:10. Antibodies to *T. gondii* were found in one of the five mice inoculated with heart of chicken no. 22 and tissue cysts were found in its brain when killed 60 days p.i. Bradyzoites released from brain tissue cysts of this isolate (designated here as TgCkNg1) were cryopreserved on March 29, 2005 and revived for the present study.

T. gondii DNA was extracted from tissues of positive mice brain using DNeasy kit (Qiagen) and genotyped using the genetic markers SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico (Dubey et al., 2006; Su et al., 2006). SAG2 was performed on all isolates, irrespective of the prior publication.

PCR products for representative strains from each genotype identified in this study were further investigated by DNA sequencing at loci SAG2, L358, PK1 and UPRT1. Two chicken isolates (TgCkGh1 and TgCkGh2) previously reported from Ghana were included in this study (Dubey et al., 2008b) to compare the DNA sequence (done for the present study) data and PCR-RFLP results (reported earlier study) with the present study. The primers used for DNA sequencing are SAG2-SqF, TAGCTTTCAAGACCGCACCT and SAG2-SqR, CTGCTTGCGATTCTGTGTGT for locus SAG2; L358-SqF, ATGTCCTCTTTCTGCCTTCG and L358-SqR, GGAGAAAGCGAAACCTTCCT for locus L358; and PK1-SqF, GGCACAATGGAAGACGATTT and PK1-SqR, GTACCAGGCCACCAAACATT for locus PK1. DNA sequence data were processed using BioEdit software, freely available at <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>. The DNA sequence lengths for SAG2, L358, and PK1 are 471, 363 and 846 bp, respectively. In addition, DNA sequences for UPRT1 (492 bp) were also generated by previously reported method (Khan et al., 2007). Phylogenetic network analysis was performed for each locus using the program SplitsTree 4 (Huson and Bryant, 2006). The sequences were submitted to Genbank.

3. Results

All the 18 groups where MAT positive and had tissue cysts in the brain by PCR-RFLP four genotypes were identified among the 19 isolates. Thirteen isolates

Table 1
Summary of genotyping results

<i>T. gondii</i> strain	Source	Genetic markers										
		SAG1	5' + 3' SAG2 ^a	SAG2 ^b	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Appico
RH88	Reference	I	I	I	I	I	I	I	I	I	I	I
PTG	Reference	II or III	II	II	II	II	II	II	II	II	II	II
CTG	Reference	II or III	III	III	III	III	III	III	III	III	III	III
TgCgCa1 (COUGAR)	Reference	I	II	II	III	II	II	II	u-1 ^c	I	u-2 ^d	I
MAS	Reference	u-1	I	II	III	III	III	u-1	I	I	III	I
TgCatBr5	Reference	I	III	III	III	III	III	I	I	I	u-1	I
TgCkEg12 ^e	Egypt	II or III	III	III	III	III	III	III	III	III	III	III
TgCkEg13 ^e	Egypt	II or III	III	III	III	III	III	III	III	III	III	III
TgCkEg14 ^e	Egypt	II or III	III	III	III	III	III	III	III	III	III	III
TgCkEg15 ^e	Egypt	II or III	II	II	II	II	II	II	II	II	II	I
TgCkEg16 ^e	Egypt	II or III	III	III	III	III	III	III	III	III	III	III
TgCkEg17 ^e	Egypt	II or III	III	III	III	III	III	III	III	III	III	III
TgCkEg19 ^e	Egypt	II or III	II	II	II	II	II	II	nd	II	II	I
TgCkNg1	Nigeria	u-1	I	II	III	III	III	III	I	I	III	I
TgCkMal-2 ^f	Mali	II or III	III	III	III	III	III	III	III	III	III	III
TgCkMal-3 ^f	Mali	II or III	III	III	III	III	III	III	III	III	III	III
TgCkMal-4 ^f	Mali	II or III	III	III	III	III	III	III	III	III	III	III
TgCkMal-5 ^f	Mali	II or III	II	II	II	II	II	II	nd	II	II	I
TgCkBF-1 ^f	B. Fasco	II or III	III	III	III	III	III	III	III	III	III	III
TgCkDROC-3 ^f	Congo	II or III	III	III	III	III	nd	III	III	III	III	III
TgCkDROC-6 ^f	Congo	II or III	III	III	III	III	III	III	III	III	III	III
TgCkDROC-8 ^f	Congo	II or III	III	III	III	III	III	III	III	III	III	III
TgCkDROC-9 ^f	Congo	II or III	III	III	III	III	III	III	III	III	III	III
TgCkDROC-10 ^f	Congo	II or III	II	II	II	II	II	II	II	II	nd	II
TgCkKen-1 ^f	Kenya	II or III	II	II	II	II	II	II	II	II	II	I
TgCkGh1 ^g	Ghana	u-1	II	II	III	III	II	II	III	II	III	I
TgCkGh2 ^g	Ghana	II or III	III	III	III	II	II	II	III	III	II	III

^a The SAG2 marker based on 5'- and 3'-end DNA sequence polymorphisms of SAG2 gene (Howe et al., 1997).

^b The SAG2 marker developed recently based on 5'-end DNA sequence of SAG2 gene is able to identify additional alleles often seen in atypical *T. gondii* strains (Su et al., 2006).

^c Unique 1.

^d Unique 2.

^e See Dubey et al. (2003) for the source chicken samples 18, 25, 26, 29, 37, 45, and 49.

^f See Dubey et al. (2005) for the source of chicken samples.

^g See Dubey (2008a) for the source of chicken samples.

(TgCkEg12, 13, 14, 16, 17, TgCkMal-2, 3, 4, TgCkBF-1, TgCkDROC-3, 6, 8, 9) belong to Type III clonal lineage. Four isolates (TgCkEg15, 19, TgCkMal-5, TgCkKen-1) that had Type II alleles at all loci except the Type I allele at Appico, and one isolate (TgCkDROC-10) had Type II alleles at all loci, all these isolates belong to the Type II clonal lineage (Table 1, Fig. 1). One genotype (TgCkNg1) shares most alleles with reference strain MAS except at locus c22-8. DNA sequencing was carried out for representative isolates including TgCkEg15 (Type II), TgCkEg16 (Type III), TgCkNg1 (atypical), TgCkGh1 (atypical) and TgCkGh2 (atypical). The results of DNA sequences are in agreement with PCR-RFLP data and further reveal that the atypical genotypes TgCkNg1 (Genbank accession nos. EU650328, EU650330) and TgCkGh1

(Genbank accession nos. EU650327, EU650329) are diverged strains. DNA sequences of TgCkGh2 match with RFLP results and are identical to the corresponding allele Type II or III sequences at a given locus, suggesting that this strain is possibly derived from recombination of II × III cross.

4. Discussion

Little is known about the genetic diversity of the African isolates. Ajzenberg et al. (2004) mentioned that the *T. gondii* strain WIK from a human patient with an unreported place in Africa was Type I based on microsatellite analysis; SAG2 typing was not done. Khan et al. (2005) reported that the strain WU05 was an exotic strain (with polymorphisms not seen in Types I,

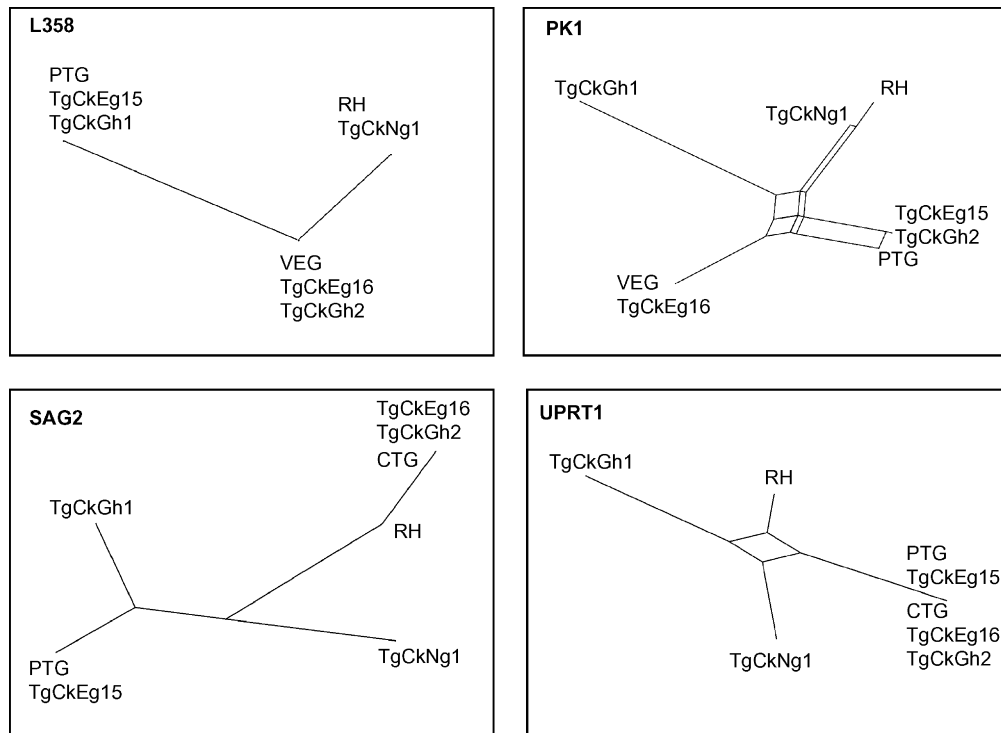


Fig. 1. NeighborNet phylogenetic network of *Toxoplasma gondii* isolates from chickens in Africa. DNA sequences at each locus were analyzed by SplitsTree 4 (Huson and Bryant, 2006). The sequence data matches with PCR-RFLP allele types perfectly at loci SAG2, L358 and PK1. At locus PK1, partial sequence for Type II strain PTG was generated, which is identical to strains TgCkEg15 and TgCkGh2. RFLP typing was not performed for locus UPRT1.

II, and III) from a human patient in USA; the patient was originally from Africa but where the person-acquired infection was unknown. Lindström et al. (2006) performed SAG2 typing on DNA isolated from blood of 27 of HIV patients with toxoplasmosis in Uganda; 18 patients had Type II, six had Type I, and three had Type III *T. gondii* DNA. In a follow up study, Lindström et al. (2008) found *T. gondii* DNA in the brains of 20 of 85 naturally exposed chickens from Uganda. Using PCR-RFLP with five markers (SAG1, SAG2, SAG3, BTUB, and GRA6) they showed that six cases were due to Type I, eight were Type II, 1 was Type III, and five chickens harbored mixed infections. Viable *T. gondii* was isolated from the tissues of nine chickens using the same procedures as in the present study. PCR-RFLP on DNA from viable parasites from these nine isolates revealed that six were Type II and three had mixed infections.

The present study is based on DNA obtained from viable isolates obtained from naturally exposed chickens from six countries in Africa. Chickens were selected for this study because they feed from ground and presumably become infected with oocysts. Detection of viable oocysts in soil is technically difficult because of the low density. Thus, the *T. gondii* isolates in chickens

are good indicators of soil contamination in a given area. Thirteen of the 19 isolates in the present study were from Egypt and DRC (Table 1) from individual homes that were at least 0.5 km apart. The three isolates from Mali were from a local market. Only single isolates were available from Nigeria, Kenya, and Burkina Faso. All of these viable isolates, and the nine isolates from Uganda (Lindström et al., 2008), were obtained in one laboratory at Beltsville and were used at their primary isolation, without many passages in cell culture or mice, thus, minimizing chances for mix up or modification of the isolates. Obtaining viable *T. gondii* isolates from Africa is technically difficult because of poor laboratory facilities in most African countries. Unfortunately, it was not possible to extend this study to more samples because of the epidemic of avian flu and restrictions imposed by the US authorities on international transport of chicken tissues.

Among 19 isolates in the present study, there were 13 Type III, five Type II and one atypical genotypes using 10 PCR-RFLP markers. Based on the present results, *T. gondii* isolates are genetically similar to those from other countries, except Brazil and Colombia. The exceptions are the Nigerian isolate TgCkNg1 and Ghana isolate

TgCkGh1 which are atypical strains. Analysis of DNA sequences showed that these strains are genetically diverged, indicating that low frequency of non-clonal lineages also circulates in Africa. DNA sequences of TgCkGh2 match with genotyping data and are identical to the corresponding allele Type II or III sequences at a given locus, indicating that it maybe derived from recombination of II \times III strains. Taken together with recent studies of *T. gondii* isolates from Africa (Lindström et al., 2008), it is clear that the same clonal lineages (Types I, II and III) predominate in North America, Europe and Africa.

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